1,1-Dichloroethylene Hepatotoxicity: Proposed Mechanism of Action and Distribution and Binding of ^{14}C Radioactivity Following Inhalation Exposure in Rats

by Rudolph J. Jaeger,[*] Lawrence G. Shoner,[*] and Linda Coffman[*]

1,1-Dichloroethylene is reported to produce renal tumors in male mice. It is an hepatotoxin in fasted rats after inhalation. We found that trichloropropane epoxide, an inhibitor of epoxide hydrase, enhances hepatic injury as measured by serum sorbitol dehydrogenase elevation. A significant elevation of hepatic citric acid concentration was seen in fasted but not fed rats. We hypothesized that mitochondrial injury was associated with inhibition of the tricarboxylic acid cycle and postulated that monochloroacetic acid was a toxic metabolite of 1,1-DCE. Fluoroacetic acid and chloroacetic acid were similar in their ability to inhibit oxygen uptake when pyruvic and malic acids were substrates in isolated mitochondria supplemented with adenosine diphosphate.

In experiments where 1,1-DCE metabolism was estimated, no difference between the rate of uptake in a 2-hr period was detected between fed and fasted animals. Urinary output of radioactivity at 26 hr for fed and fasted rats was similar. Water-soluble (i.e. TCA-soluble) 1,1-DCE metabolites were found in tissues of fasted rats in excess of that seen in fed rats. The kidney had the largest concentration of total metabolites. Tissue-bound, or TCA-insoluble, radioactivity was associated with the mitrochondrial and microsomal fraction of fasted rats in excess of that seen in fed rats. The disappearance of TCA-insoluble radioactivity from the mitochondrial and microsomal fractions was comparable in rate between fed and fasted rats respectively. These results suggest that 1,1-DCE is metabolized quite rapidly in the organism to TCA-soluble components which are excreted by the kidneys. Metabolites of 1,1-DCE may enter the metabolic pool, since a reasonably short turnover of ^{14}C-labeled, bound material was observed. The metabolite of 1,1-DCE appears to inhibit the mitochondria so that citric acid accumulates. This may occur by a process of lethal synthesis.

Introduction

1,1-Dichloroethylene (1,1-DCE, vinylidene chloride) has recently been reported by Maltoni to produce renal tumors in mice following inhalation exposure (*1*). These observations follow the earlier revelation that vinyl chloride, a closely related compound, is a hepatocarcinogen in man and experimental animals (*2*). By analogy, therefore, vinylidene chloride should be regarded as a sus-

pected human carcinogen. The toxicology of 1,1-DCE has been reported by Irish (*3*) and Prendergast et al. (*4*). Their observations demonstrate clearly that the compound produces hepatic and renal damage following inhalation exposure. No further work was reported in the literature until the reports of Jenkins et al. (*5, 6*), which attempted to elucidate some of the factors which altered the toxicity of 1,1-DCE. More recently, work from our laboratory at the Harvard School of Public Health has attempted to elucidate the mechanism of action of this acutely injurious plastics monomer (*7–13*). 1,1-DCE has been described by Reynolds as an "exquisite toxin" producing injury unlike that seen

*Toxicology Unit, Department of Physiology, Kresge Center for Environmental Health, Harvard School of Public Health, 665 Huntington Ave., Boston, Massachusetts 02115.

with a number of other chlorinated compounds (14) and with a rapidity that suggests a specific and definable lesion.

This report presents information from our laboratory on the mechanism of 1,1-DCE-induced hepatotoxicity and the reason for the potentiation of this injury by fasting and also attempts to provide a framework in which results on tissue distribution and binding of radioactivity may be related to the proposed mechanism of action. This and previous work will be used in an attempt to explain the mechanism by which injury is produced.

Materials and Methods

Male Holtzman rats (200-350 g) were used for these studies. The rats were housed in air-conditioned quarters and supplied with Purina rat chow and water *ad libitum*. Lights were on from 6 AM to 6 PM. Where indicated, rats were fasted for 18 hr prior to exposure or briefly prior to treatment. Inhalation exposures were done as previously described (10). Trichloropropane epoxide, an epoxide hydrase inhibitor (15), was administered as a 1 or 10% solution in 0.5% methyl cellulose, 0.5% Tween 80. Serum sorbitol dehydrogenase activity was measured as described by Korsrud et al. (16). Citric acid concentrations in the liver were measured according to the method of Henry (17). Oxygen uptake was measured by using a Yellow Springs Oxygraph. Oxygen consumption was determined using a standard curve supplied with the instrument. Protein concentration was determined according to the method of Lowry (18). Chemicals and reagents were of the highest purity available.

^{14}C-labeled 1,1-DCE was purchased from the New England Nuclear Company. Funds for this purchase were the generous gift of the Dow Chemical Company. Tissue fractions were prepared according to the method of Hook et al. (19). Radioactivity was measured in the TCA-soluble and TCA-insoluble precipitates. Where indicated, precipitates were extracted exhaustively with chloroform. Samples were digested using a tissue solubilizer purchased from New England Nuclear Co.; they were decolorized by use of hydrogen peroxide. Radioactivity was determined using a Searle Isocap 300 refrigerated scintillation spectrometer. DPM were determined using an external standard via the channels ratio method.

Statistical analysis was done by using the Student's *t*-test for significance of differences and a *p* value < 0.05 was considered significant.

Results

Since our earlier observations have shown that 1,1-DCE hepatotoxicity was diminished in animals pretreated with inducers of the mixed function oxidase system, we attempted to ascertain if metabolism might be important in the toxicity of 1,1-DCE. Such efforts require the direct measurement of 1,1-DCE biotransformation. Lacking this capability initially, we attempted to investigate if a potential intermediate of mixed function oxidase activation of 1,1-DCE, namely an epoxide, might exist. Data in Table 1 show that trichloropropane epoxide (TCPE), an epoxide hydrase inhibitor (15), is able to increase significantly the toxicity of 1,1-DCE in fasted rats. At constant dose, enhancement of toxicity is proportional to the concentration of the TCPE administered, and suggests that the action of TCPE may be competitive. Larger doses of TCPE are lethal but not hepatotoxic. It should be noted that we also observed a slight, but not significant, reduction of hepatic glutathione concentration in animals pretreated with TCPE (see discussion). The possibility exists that this decrease in hepatic glutathione may have produced the enhancement of 1,1-DCE toxicity that was observed.

Table 1. Effects of trichloropropane epoxide on the toxicity of 1,1-DCE.[a]

| | Serum sorbitol dehydrogenase activity, at TCPE (0.1 ml/kg Po concentration, IU/ml | | |
Exposure	Vehicle	TCPE	
Air control	8.3 ± 1.7	5.7 ± 0.3	5.4 ± 0.7
	n = 14	n = 11	n = 7
1,1-DCE	62.0 ± 12.0[b]	142.0 ± 5.0[c]	536.0 ± 125.0[c]
	n = 19	n = 16	n = 6

[a]Exposure: Holtzman rats, fasted, 100–150 ppm DCE × 4 hr; killed at 6 hr.
[b]*p* < 0.05 compared to air control.
[c]*p* < 0.05 compared to air control or vehicle-treated, 1,1-DCE exposed group.

Henschler has suggested (20) that 1,1-DCE may be converted to chloroacyl chloride, which would rearrange to monochloroacetic acid. We tested the possibility that monochloroacetic acid might result in mitochondrial injury and lead to the accumulation of citric acid within the livers of animals exposed to 1,1-DCE. This hypothesis follows the lethal synthesis hypothesis of Peters (21) that fluoroacetic acid is converted to fluorocitric acid and this compound inhibits the enzyme aconitase. Citric acid accumulates as a result. Data in Table 2 show that animals exposed to 1,1-DCE following 18 hr fast did indeed have a highly significant elevation (416%) of liver citric acid and a 636.4-fold increase in SDH activity. Liver citric acid levels in fed animals were higher but, following exposure, citric acid concentrations were not significantly elevated (122% of control). A 100-fold elevation of SDH activity was observed in these animals.

Table 2. Effect of Air or 1,1-Dichloroethylene exposure on hepatic citric acid concentrations and serum sorbitol dehydrogenase activity.[a].

	Citric acid, mg/100 g	Sorbitol dehydrogenase, IU/ml
Fasted, air-exposed	8.33 ± 1.07 ($n = 9$)	5.0 ± 0.5 ($n = 10$)
Fasted, 1,1-DCE-exposed	34.71 ± 4.32 ($n = 10$)[b]	3181.9 ± 674.8 ($n = 9$)[b]
Change, μl	416	636.4-fold
Fed, air-exposed	22.74 ± 1.26 ($n = 10$)	5.4 ± 0.8 ($n = 10$)
Fed, 1,1-DCE-exposed	27.89 ± 2.96 ($n = 10$)	539.1 ± 163.3 ($n = 10$)[b]
Change, μl	122	99.8-fold

[a]Exposure: Holtzman rats, 250 ppm 1,1-DCE $\times$ 4 hr; killed at 6 hr.
[b]$p < 0.05$ compared to corresponding control.

In order to estimate the toxicity of chloroacetic acid as a metabolite of 1,1-DCE, we conducted a series of LD_{50} determinations using 2-fluoroethanol, 2-chloroethanol and 2,2-dichloroethanol in fed or fasted Holtzman rats. We attempted to determine if there would be a fed/fasted difference in toxicity for these halo acid precursors. No such difference was found. We observed that the monochloroethanol LD_{50} was between 0.6 and 1.05 mmole/kg, while the LD_{50} for fluoroethanol was between 0.02 and 0.08 mmole/kg, an approximately 10-fold difference. The LD_{50} of dichloroethanol was in excess of 8 mmole/kg. The monochloroethanol and monofluoroethanol LD_{50} values compare favorably with those reported by Hayes et al. (22) for monochloro- and monofluoroacetic acids, respectively.

Table 3 shows the effect of fluoroacetic acid incubation on the oxygen uptake of mitochondria incubated alone or with pyruvic and malic acids as substrate and adenosine diphosphate as phosphate acceptor (state III conditions). Similar data for chloroacetic acid are shown in Table 4. It can be seen that fluoroacetic acid (Table 3) results in a significant increase of oxygen uptake by mitochondria in the absence of substrate at 2 and $5 mM$ fluoroacetic acid. A similar, significant, elevation was observed with chloroacetic acid (Table 4) at 5 mM. In the presence of substrate, both fluoroacetate and chloroacetate resulted in an inhibition of the enhanced oxygen uptake that was observed in mitochondria alone (92% and 110% compared to 149%, fluoroacetate; 164% and 158% compared to 178%, chloroacetate). Fluoroacetate was much more effective at inhibiting oxygen uptake in the presence of substrate than was chloroacetate. In the presence of both substrate and ADP, both fluoroacetate and chloroacetate significantly inhibited the increased oxygen uptake that was associated with state III conditions. Again,

Table 3. Effect of fluoroacetic acid (FA) preincubation on the oxygen uptake of mitochondria incubated with pyruvic—malic acid (substrate) and adenosine diphosphate (ADP).

FA, mM	Oxygen uptake, nmole O_2/min-mg protein[a]			Mitochondria control activity, %		
	Mitochondria alone	Mitochondria + substrate	Mitochondria + substrate + ADP	Mitochondria alone	Mitochondria + substrate	Mitochondria + substrate + ADP
0 (control)	1.47 ± 0.25	2.20 ± 0.37	2.84 ± 0.67	100	149	193
2.0	2.57 ± 0.21[b]	1.35 ± 0.13[b]	0.91 ± 0.11[b]	174	92	61
5.0	2.49 ± 0.18[b]	1.62 ± 0.33	1.19 ± 0.29[b]	169	110	80

[a]Values shown are the means $\pm$ SEM of 6 determinations per group.
[b]Significantly different from corresponding control activity.

Table 4. Effect of chloroacetic acid (CA) preincubation on the oxygen uptake of mitochondria incubated with pyruvic–malic acid (substrate) and adenosine diphosphate (ADP).

CA, mM	Oxygen uptake, nmole O_2/min-mg protein[a]			Mitochondria control activity, %		
	Mitochondria alone	Mitochondria + substrate	Mitochondria + substrate + ADP	Mitochondria alone	Mitochondria + substrate	Mitochondria + substrate + ADP
0 (control)	1.12 ± 0.23	2.00 ± 0.27[b]	2.54 ± 0.21[b]	100	178	227
2.0	1.65 ± 0.17	1.83 ± 0.18	1.43 ± 0.36[b]	147	164	128
5.0	2.60 ± 0.76[b]	1.77 ± 0.27	1.56 ± 0.29[b]	232	158	139

[a]Values shown are the means $\pm$ SEM of 6 determinations per group.
[b]Significantly different from corresponding control activity.

fluoroacetic acid was quantitatively more effective at inhibiting oxygen uptake. These results are suggestive that fluoroacetate and chloroacetate are similar in their ability to inhibit oxygen uptake of mitochondria under state III conditions. This observation supports but does not prove our hypothesis that chloroacetic acid, by a process of lethal synthesis, is converted into monochlorocitric acid, which inhibits the enzyme aconitase.

In Figure 1, the recirculating chamber that was used in our studies is illustrated. This chamber design permitted four rats to be exposed simultaneously. Disappearance curves for 1,1-DCE from this chamber are shown in Figure 2. It can be seen that a very slight, nonsignificant decrease does occur over the 120-min time period given an empty chamber or a chamber containing four dead rats. In live fed or fasted rats, the data show clearly that there is no significant difference between the rate of uptake of 1,1-DCE.

In Figure 3, the distribution of ^{14}C radioactivity in various organs, 30 min following a 2 hr exposure of fed or fasted rats, is shown. There was no significant difference between the amounts of radioactivity found in brain from fed or fasted rats. A slightly higher content of total radioactivity was seen in heart from fasted rats. Spleen and sera demonstrated that a significant increase in total radioactivity could be observed in fasted rats. The largest amount of total radioactivity was detected in the kidneys of fasted rats. The majority of this was water- or TCA-soluble. In the liver, a significant and larger portion than that radioactivity found in kidney was in the TCA-insoluble form, representing either ^{14}C tightly bound to tissue constituents, or ^{14}C that had entered the metabolic pool. In contrast, the large amount of radioactivity found in the kidneys of fasted rats at this time suggests that a substantial amount of metabolism of 1,1-DCE has occurred, and this metabolism produces a TCA-soluble product which is excreted via the kidney.
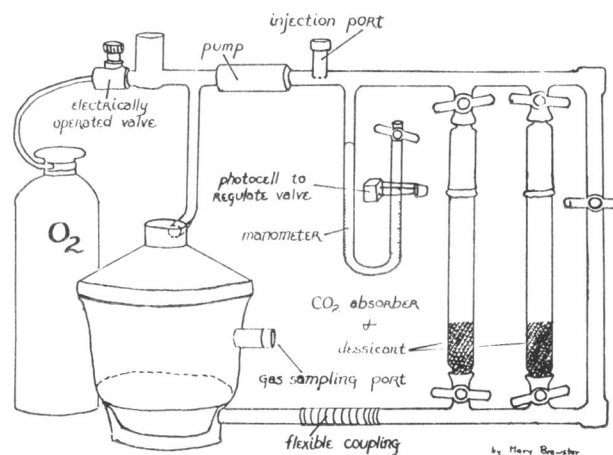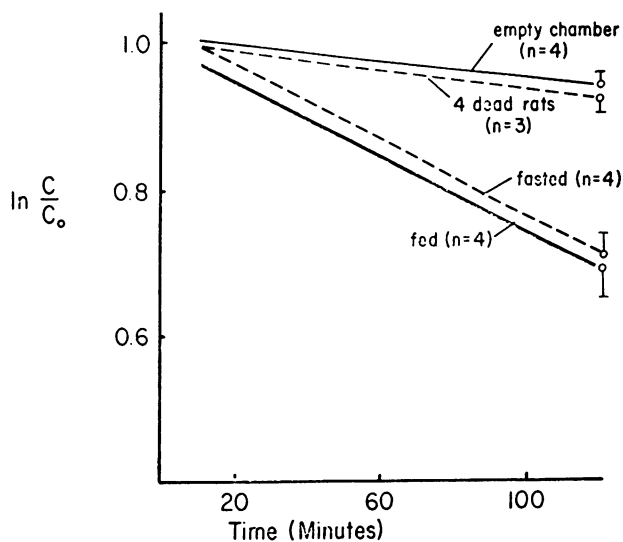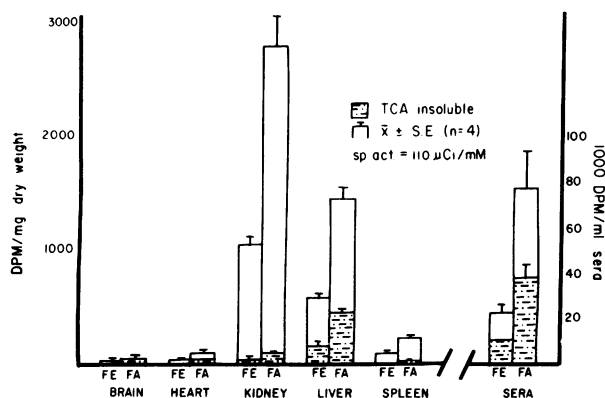

FIGURE 1. Recirculating exposure chamber.

FIGURE 2. Disappearance curves for 1,1-DCE. Initial concentration was ca. 2000 ppm. Each experiment was conducted with four rats in the chamber. Line is calculated as the line of best fit by the least squares method.

FIGURE 3. Distribution of ^{14}C radioactivity in various organs. Exposure at $t = 0$ was 2000 ppm and the rats were killed at 30 min. The exposure was terminated by venting the chamber in a hood.

In Figure 4, the subcellular distribution of ^{14}C in the liver 30 min following a 2-hr exposure, is shown. In all liver fractions from fasted animals, significantly more radioactivity was found than in similar fractions from fed animals. The radioactivity in mitochondria and microsomes from fed or fasted rats is largely in the TCA-insoluble fraction. The cytoplasm fraction contained substantial amounts of TCA-soluble radioactivity, suggesting that a metabolite was present. In the mitochondrial and
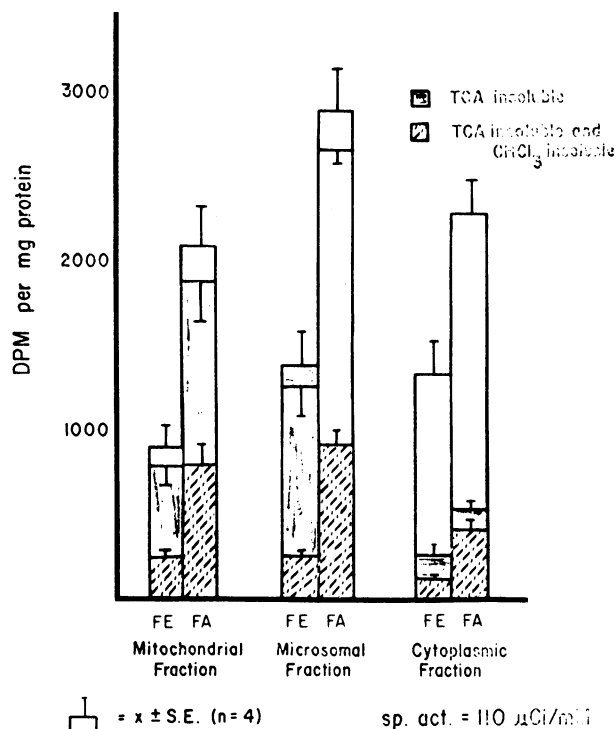
FIGURE 4. Subcellular distribution of ^{14}C activity in liver. Experimental details given in legends to Figs. 1–3 and text.
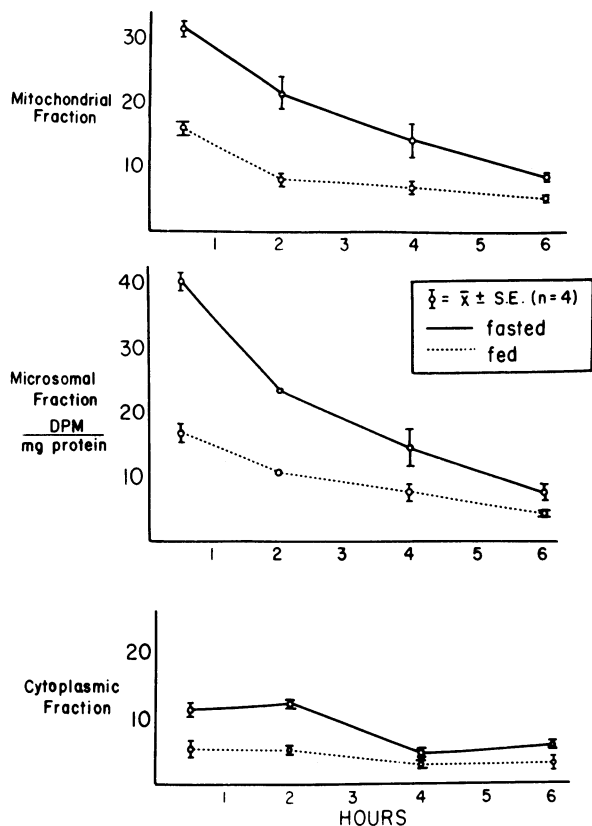

FIGURE 5. Disappearance curves of TCA-insoluble ^{14}C activity from subcellular fractions of liver. These data correspond to the experiment shown in Fig. 2 using animals killed at the time indicated. See text for details.

microsomal fraction this radioactivity accounted for a minority of the ^{14}C activity that was detected. Significant amounts of the radioactivity in these two fractions was chloroform-soluble, suggesting that lipid binding of ^{14}C might constitute a substantial site of loss for the radiolabel.

The disappearance curves of TCA-insoluble ^{14}C radioactivity from subcellular fractions of the liver are shown in Figure 5. It can be seen that the mitochondrial and microsomal fractions from fasted rats, which contained significantly more activity than fed rats, had approximately the same time course as that seen in fed animals: a $T_{1/2}$ in the vicinity of 2–3 hr. The amount of radioactivity in the cytoplasmic fraction was reasonably constant and higher in fasted animals. These results suggest that while more TCA-insoluble radioactivity is found in the mitochondrial and microsomal fractions of livers from fasted animals, the turnover time is rapid and may represent radioactivity which has entered the metabolic pool rather than TCA-insoluble radioactivity covalently bound to protein or other tissue constituents.

Table 5 shows the excretion of ^{14}C radioactivity into the urine. Groups of four fed or fasted rats were exposed to 1,1-DCE (^{14}C) for 2 hr at an initial concentration of 2000 ppm and the animals were

killed 24 hr after exposure. The data show that of the calculated total dose absorbed, 36.7% and 36.5%, was recovered in the urine of fed rats and fasted rats, respectively. When the chemical distribution of radiolabel in the urine was determined, no fed or fasted differences were seen in ether-extractable fractions from acidified urine. Very little radioactivity was chloroform-extractable from the alkaline urine. These results suggest that differences in metabolite distribution do not exist between fed and fasted rats, and excess of one metabolite over another does not explain the toxicity differences that are observed.

Table 5. Excretion of ^{14}C radioactivity in urine.[a]

	Fed	Fasted
Total DPM in 26-hr urine	594,048 ± 48,205	638,675 ± 59,025
% of total dose	36.7 ± 3.3	36.5 ± 4.1

[a]Groups of four fed or fasted rats were exposed to ^{14}C-1,1-DCE for 2 hr at an initial concentration of 2000 ppm and sacrificed 24 hr after exposure.

Discussion

In the present report we have shown that the toxicity of 1,1-DCE is modified when animals are pretreated with trichloropropane epoxide. This suggests but does not prove the involvement of an epoxide intermediate in the toxicity of 1,1-DCE. There currently exists no information on the metabolism of vinyl chloride or vinylidene chloride epoxides by the enzyme epoxide hydrase. If it is shown that epoxide hydrase is unable to hydrate such small epoxides such as result from vinyl chloride or vinylidene chloride metabolism, then an alternate explanation for our observation must be found. As noted under results, we observed that glutathione concentration was decreased 18% below control value following the administration to fasted rats of TCPE at a concentration of 10% in an oral dose of 0.1 ml/kg (4.76 ± 0.50 mg GSH/100 g in H$_2$O control vs. 3.90 ± 0.42 mg GSH/100 g in the TCPE group). This decrease, although not statistically significant between groups, may have accounted for the enhanced toxicity that followed combined TCPE and 1,1-DCE exposure.

Some controversy exists on the role of the MFOS in the activation of 1,1-DCE. Should epoxide hydrase not be involved, our indirect evidence of MFOS activation would be weakened. We reported (11) that the MFOS inhibitor SKF 525 A did not decrease the toxicity of 1,1-DCE (1000 ppm × 4 hr, fasted rats). In subsequent experiments reported in 1975 but as yet unpublished (12) we showed that a larger dose of SKF 252 A (75 mg/kg, IP) did protect against the toxicity of 1,1-DCE (200 ppm × 4 hr). The expected elevation of serum transaminase in vehicle-treated, fasted rats exposed to 1,1-DCE was decreased to one half by treatment with this inhibitor of MFOS. Further, VCM, which is known to be activated by the MFOS, competitively blocked the toxicity of 1,1-DCE at concentrations of 200–2000 ppm (11), conditions where VCM metabolism is saturated (23).

Our determination that hepatic citric acid levels became elevated following exposure to 1,1-DCE suggests that 1,1-DCE affect the mitochondria with an attendant inhibition of the tricarboxylic acid cycle. This postulation of mitochondrial damage is consistent with our previously-reported morphologic observations (14). Our hypothesis, based on the reports of Henschler (20) and Hathway (24) that chloroacyl chloride is converted into monochloroacetic acid, which may, by a process of lethal synthesis, be converted into chlorocitric acid which inhibits the enzyme aconitase, is shown by the similar effects between monochloroacetic acid and monofluoroacetic acid. Both compounds inhibit

oxygen uptake in isolated mitochondria when pyruvic and malic acids are used as the substrate.

In preliminary experiments, using isolated mitochondria with succinate as the substrate, we are unable to show an inhibitory effect of monochloroacetic acid on oxygen uptake. When acetic acid was used as substrate, oxygen uptake was inhibited (25). These results suggest that whatever inhibition has occurred is at an enzymatic site prior to the metabolism of succinate. It should be noted that we have found that oxygen uptake of whole-liver homogenates from animals treated with 1,1-DCE and supplemented with succinate is inhibited (13). This result suggests that the inhibitory effect of 1,1-DCE on mitochondria is more complex than merely a blockage or inhibition at some step in the TCA cycle. Our observation of oxygen uptake inhibition is consistent with the report of Hayes et al. (22) suggesting that monochloroacetic acid toxicity was similar in some respects to monofluoroacetic acid, i.e., inhibition of ^{14}C acetate metabolism. The observation was made by them that sufficient differences existed to suggest that monochloroacetic acid has a different mechanism of toxicity from monofluoroacetic acid. It should be noted that monochloroacetic acid is a direct alkylating agent (26).

With regard to our observations on the uptake of 1,1-DCE in fasted rats versus fed rats, it appears that, if uptake from the recirculating exposure chamber is an accurate measure of metabolism *in vivo*, there is no difference due to fasting in the rate of metabolism under these circumstances. Further, the similarity of 24-hr output of radioactivity following exposure suggests that overall there is no difference in the amount of metabolism.

We did measure significantly more water-soluble ^{14}C radioactivity in the kidneys of fasted rats at 30 min following a 2-hr exposure. A significantly greater amount of water-soluble radioactivity was found in the liver cytoplasm from fasted rats. This suggests that while no significant differences in metabolism exist between the fasted and fed rat as reflected by uptake from the recirculating chamber or 26-hr output, there does appear to be a significant difference of pathway chosen *in vivo* for the products of metabolism. Further, the observation of substantially larger amounts of ^{14}C radioactivity in the kidneys at early times of exposure suggests that concentration differences may exist between the liver and kidneys in these two treatment groups.

In conclusion, we suggest that an epoxide intermediate may be formed upon 1,1-DCE metabolism in the liver. Based on the reports of Reichert and Bashti and others (20, 24, 27) we believe that monochloroacetic acid may be the toxic metabolite

responsible for the observed toxicity. Our results are not inconsistent with the results of Hayes et al. (22) and the statement by Van Duuren et al. (26) that monochloroacetic acid is a direct alkylating agent.

Lastly, the acute toxicity of 1,1-DCE may be such that liver cells are destroyed rather than transformed by this compound; thus hepatocellular carcinogenesis may be an unlikely event due to acute cellular toxicity. However, the production of an alkylating agent and its concentration by the kidney, as well as species differences between rats and mice, could be a likely explanation for the observation that mice do develop renal tumors following long-term exposure to 1,1-DCE while rats only show significant hepatocellular injury.

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